High-Pressure Liquid Chromatography in the Separation and Detection of Bitter Compounds

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The constituents present in hops which are responsible for the characteristic bitter flavor of beer consist of a complex mixture of closely related compounds. Separation and analysis of these compounds by countercurrent distribution, the best technique available, is slow and tedious. High-pressure liquid chromatography (hplc) has therefore been investigated as an alternative method for performing rapid analyses. The technique shows considerable potential for separation and detection of bittering constituents in both hops and beer. Hplc has also been used to follow the rearrangement of hop components and to monitor synthetic reactions.

Hops, the dried female inflorescence of the hop plant, *Humulus lupulus* L., are used in the brewing process to impart a desirable bitter flavor and aroma to the finished beer. The bitter compounds are formed, during boiling of the hops in wort, from the resinous constituents secreted by the lupulin glands at the base of the bracts. The complex chemistry of the hop resins has fascinated organic chemists for over 60 years and has been the subject of excellent reviews by Stevens (1967) and Ashurst (1967).

HOP RESIN CONSTITUENTS

The resin fraction of hops consists of two major groups of compounds, the α acids (humulones) and β acids (lupulones), which together normally comprise 10–15% of the weight of the dried hops, depending upon variety. These compounds are of mixed biogenetic origin and may be regarded as phloracylphenones, modified by introduction of isopentenyl side-chains and derived biogenetically from the desoxy α acids. The biosynthetic pathways, by which the hop plant produces the α and β acids, follow a common route to the desoxy α acids, at which point they divide to give α acids by oxidation and β acids by further substitution with an isopentenyl group (Figure 1). As a result, the amount of desoxy α acids present in the hop never becomes large, since they are siphoned off as they are formed to give the end products of the biosynthetic pathway, the α and β acids, which increase in quantity as the hop matures.

Both α and β acid fractions of hops consist of a mixture of homologs in which the structure of the acyl side-chain varies. Thus, the α acid fraction consists of humulone, cohumulone, adhumulone, posthumulone, and prehumulone, together with trace quantities of other unnamed homologs. A similar series of lupulone homologs comprises the β acid fraction.

The proportionate composition of the α and β acid fractions varies widely with the variety of hop. Kowaka *et al.* (1970) have determined these differences in terms of percentage composition *vs.* hop variety, and the results are shown in Table I. European hops generally contain large quantities of humulone in relation to the other homologs, whereas American and Japanese varieties frequently contain almost as much cohumulone as humulone. These differences may be quite significant, since cohumulone is normally better utilized in the brewing process and may give rise to less desirable flavor characteristics than humulone (Rigby, 1972). A similar relationship applies to the β acid fraction, although the levels of colupulone tend to be much greater than those of the corresponding homolog in the α acid series.

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Since only the α acid fraction is utilized during the brewing process, with the β acids remaining essentially unchanged, it is obviously desirable to have a rapid analytical method available to determine the relative percentages of each group in a given hop sample. In the past such analyses have been carried out by polarimetric, conductometric, and spectrophotometric methods, the latter being most commonly used.

The recent development of high-pressure liquid chromatography (hplc) as an analytical tool has provided an alternative method for separation and estimation of resin constituents present in hops and beer. The usefulness of gas chromatography in this regard has been somewhat limited by the necessity of preparing trimethylsilyl ethers and using all-glass systems to avoid decomposition of these derivatives (Segel and Molyneux, 1971). Moreover, the tautomerism of these compounds may lead to the formation of more than one derivative from each constituent. The use of tlc for qualitative determination of hop constituents (Grant, 1970) indicated that the most suitable packing material for hplc would be of the silica gel type. A stainless steel column, 1 m long and 2 mm i.d. packed with Corasil II, and a solvent system consisting of various proportions of isooctane–ethyl acetate, delivered at *ca.* 180 psi by a pulseless pump, were found to be most generally applicable to the separations desired. Monitoring of the effluent was most conveniently achieved using a uv detector at 280 mμ, a wavelength at which most hop constituents show strong absorption.

Under the above conditions, a synthetic mixture of pure humulone and colupulone was separated completely in less than 6 min. A comparable separation of the α and β acids in an isooctane extract of hops was achieved similarly (Figure 2), thus providing a rapid method for the determination of these constituents in hops or hop extracts. As yet it has not been found possible to separate the individual homologs of either the α or β acid fraction by hplc.

In order to test the separative power of the hplc system, a mixture of colupulone and its hexahydro derivative, in which the double bonds of the isopentenyl side-chains have been hydrogenated over Adam's catalyst, was run. In this case separation of the two components was achieved using a Sil-X packing with isooctane–chloroform as the solvent (Figure 3).

It can be seen from Figure 1 that the unused β acids could potentially be converted to the α acids, which are required in the brewing process, *via* cleavage of one of the *gem*-isopentenyl side-chains to the desoxy α acids and subsequent oxidation to the α acids. In fact the first step of this route can be achieved quite readily by a photochemical reaction discovered by Fernandez (1967). The progress of the reaction is normally followed by the change in the uv spectrum of the solution but hplc monitoring of the reaction is equally convenient. Figure 4 dem-

Table I. Variation of α and β Acid Composition with Hop Variety Composition, %

Hops	α acids			β acids		
	Humulone	Cohumulone	Adhumulone	Lupulone	Colupulone	Adlupulone
Japanese	46	41	13	21	68	11
American	54	34	12	32	57	11
Hallertau	59	27	14	45	43	12
Northern Brewer	64	24	12	46	43	11
Saaz	67	21	12	51	37	12

onstrates such an analysis at the commencement of the reaction when only colupulone is present and at the end of the reaction when desoxycohumulone and a second unidentified product can be detected.

HUMULONE-ISOHUMULONE ISOMERIZATION

The most important chemical reaction of the hop resins in the brewing process is the isomerization of the α acids to iso-α acids. This transformation gives rise to the majority of the bitter substances present in beer and, in consequence, has a great influence on the taste of the finished product.

During the isomerization, each α acid forms two compounds having the same chemical structure, but differing in the stereochemical arrangements of two of the sidechains attached to the basic ring structure (Figure 5).

It was believed for some time that these two compounds, the *cis-* and *trans*-isohumulones, had different bittering potential, but Verzele *et al.* (1970) have recently shown that they are essentially equally bitter and the relative quantities of cis and trans isomers produced are, therefore, unimportant as far as bittering power is concerned.

Three different methods are available for carrying out the humulone-isohumulone isomerization. 1. Heating humulone in aqueous acid or base solution at pH 4.9–10.9. This method approximates the wort-boiling step in the brewing process, which is generally in the pH range 4.9–5.4. 2. The magnesium ion catalyzed method, developed by Koller (1969), which is carried out at essentially neutral pH. 3. The photochemical method of Clarke and Hildebrand (1965), which involves irradiating a methanol so-

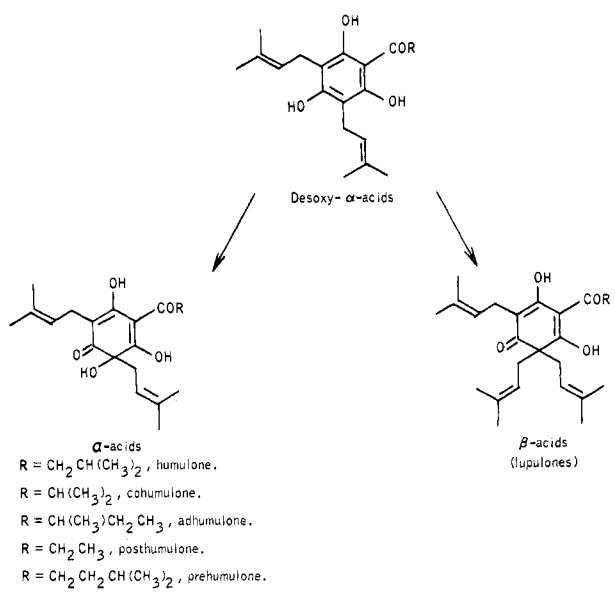


Figure 1. Interrelationship of α and β acids.

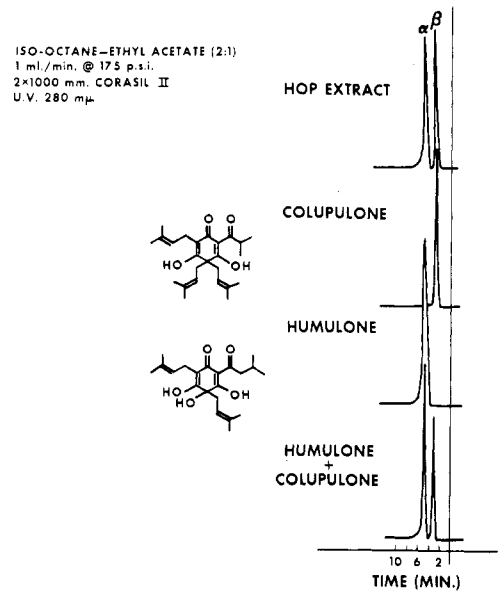


Figure 2. Separation of α and β acids by hplc.

lution of humulone with ultraviolet light. The first two methods give a preponderance of the *cis*-isohumulone, whereas the third method yields a larger amount of the trans isomer (Figure 5). The three methods give different overall yields of isohumulone, the first method tending to give lower yields at the more extreme pH values due to hydrolysis of isohumulone to humulinic acids, a process which does not occur readily with methods 2 and 3.

It has recently been found that the *cis-* and *trans*-isohumulones are not directly interconvertible, but that the isomerization process is a reversible process in which humulone can be reformed from each isomer (Aitken *et al.*, 1969). The transformation of the cis to the trans isomer and *vice versa* is therefore not very facile, and the *cis–trans* ratio, once established, is maintained. The two isomers do not degrade to the respective *cis-* and *trans*-humulinic acids at the same rate, with the *cis*-isohumulone undergoing decomposition much more rapidly than the trans isomer (Figure 6). In consequence, an isohumulone mixture containing a high percentage of the trans isomer would be much less susceptible to loss of bitterness by transformation into nonbitter humulinic acids than a mixture with a high cis isomer content. For this reason an attempt should be made, during the formation of isomerized hop extracts, to obtain the product with the highest proportion of trans isomer possible.

It is obviously important to be able to determine with accuracy the extent of the humulone-isohumulone isomerization, particularly during the preparation of preisomerized hop extracts. Hplc has been applied to the monitoring of the photochemical conversion of humulone into isohumulone, as illustrated in Figure 7. A sample taken shortly after commencing irradiation shows mainly un-

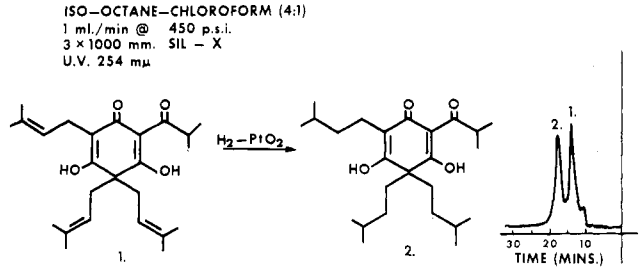


Figure 3. Separation of colupulone and hexahydrocolupulone.

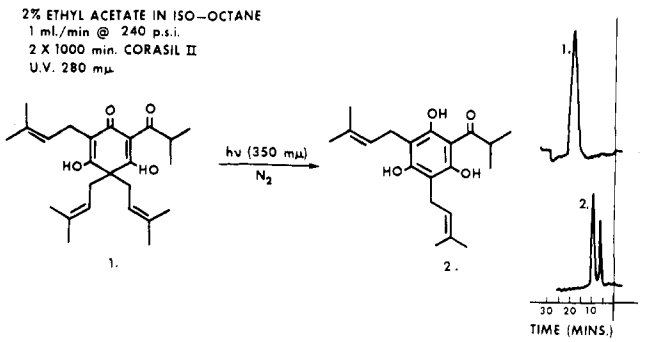


Figure 4. Separation of colupulone and desoxy-cohumulone.

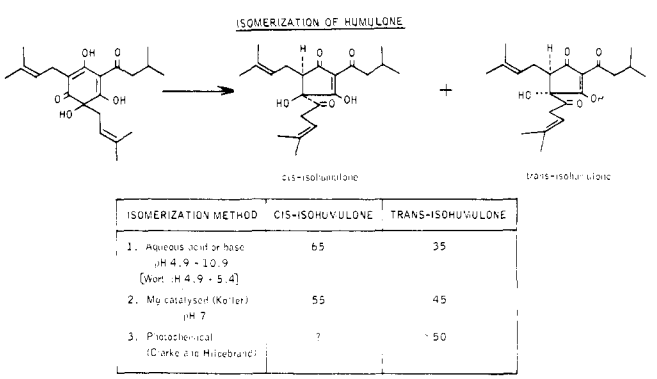
changed humulone, whereas a sample taken near completion of the reaction shows isohumulone with small amounts of starting material remaining. Under these conditions the *cis*- and *trans*-isohumulones are eluted simultaneously. As yet conditions for the separation of isohumulones and humulinic acids have not been worked out, but such an analysis would be of value in determining the amount of loss of bittering compounds through degradation to nonbitter constituents.

ANALYSIS OF ISOHUMULONES IN BEER

The desirable bitter flavor of beer does not result from the presence of a single compound derived from hops. Many compounds may be present which, although not as bitter as the isohumulones, can have the effect of modifying the overall flavor pattern. Even if the oxidized hop resins are excluded from consideration as flavor contributors, the isohumulones, humulinic acids, and *allo*-isohumulones in which the double bond of the isopentenoyl sidechain has moved into conjungation with the carbonyl group comprise a large collection of closely related compounds.

Obviously, the analysis of such a complex mixture presents a formidable task and the only technique capable of approaching the problem has been gas chromatography of the volatile trimethylsilyl ether derivatives. This technique has been used to distinguish between different hop varieties used for flavoring beer (Molyneux and Eggling, 1971). However, the separative power of hplc, combined with its other advantages, makes it ideally suited for analyzing for hop constituents in beer. Thus it is not necessary to prepare volatile derivatives; samples of the separated constituents can be readily collected for analysis by physical methods and separations can be altered readily by variation of the solvent mixture and column packing.

The results of preliminary experiments using hplc for separation of hop constituents in beer are illustrated in Figure 8. The isooctane extract contains the isohumulones and most of the nonoxidized hop resin constituents, since



ISOMERIZATION METHOD	CIS-ISOHUMULONE	TRANS-ISOHUMULONE
1. Aqueous acid or base pH 4.9 - 10.9 [Wort pH 4.9 - 5.4]	65	35
2. Mg catalyst (Kolter) pH 7	55	45
3. Photochemical (Clarke and Hricebrach)	?	~50

Figure 5. Isomerization of humulone.

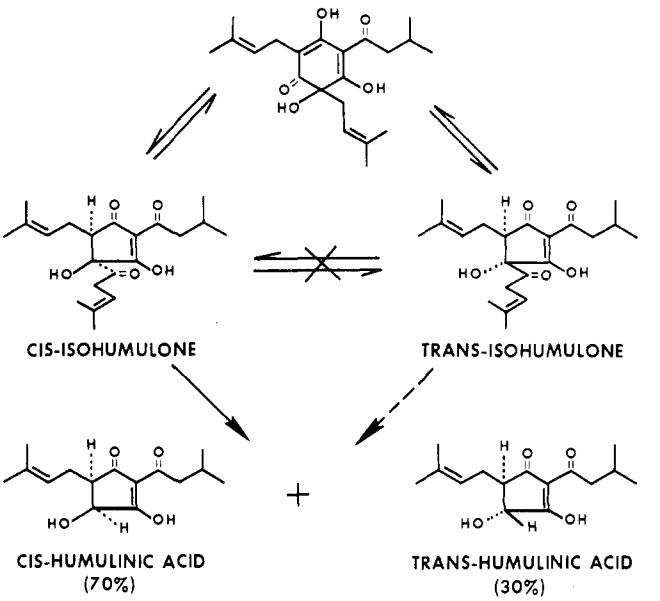


Figure 6. Transformation of humulone to humulinic acids.

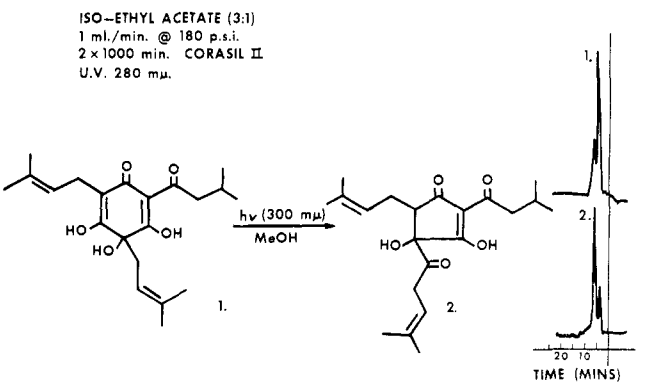


Figure 7. Separation of humulone and isohumulone.

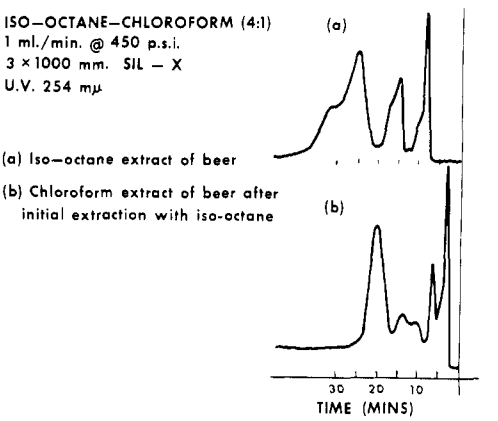


Figure 8. Hplc analysis of beer extracts.

an extract of an unhopped beer shows no constituents on either glc or hplc analysis. The chloroform extract contains mainly oxidized hop resins together with non-hop-derived compounds. Obviously, the separations achieved with such beer extracts using a single column packing and solvent system cannot be expected to resolve each of the hop components but rapid separation into the major fractions is achieved and each of these fractions can be subjected to further analysis. The success of Kokubo *et al.* (1971) in analyzing beer extracts by regular column chro-

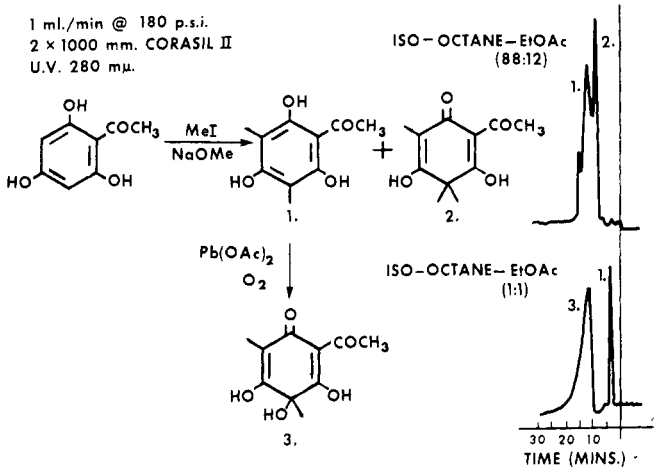


Figure 9. Separation of 3,5-dimethyl-, 3,3,5-trimethyl-, and 3,5-dimethyl-3-hydroxy-phloracetophenone.

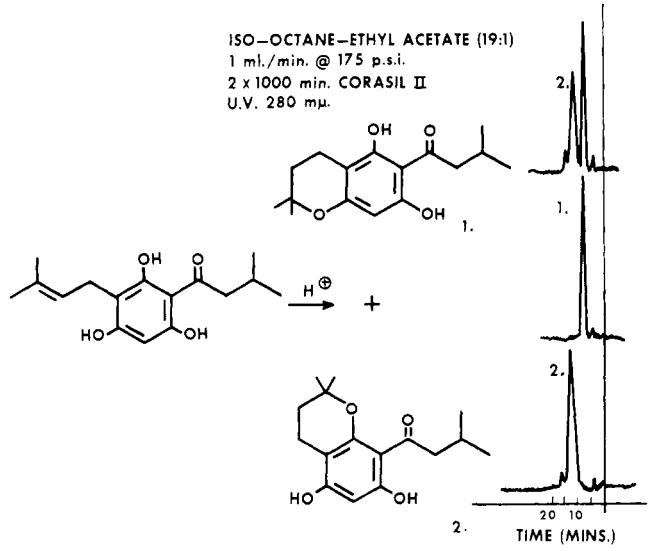


Figure 10. Separation of 6- and 8-isovaleryl-5,7-dihydroxy-2,2-dimethylchroman.

matography on ion-exchange resins suggests that the application of hplc separations on suitable ion-exchange packings would give rapid and complete analyses.

HPLC OF SYNTHETIC COMPOUNDS

High-pressure liquid chromatography is ideally suited for analysis and separation of complex mixtures formed during the synthesis of model compounds which have been prepared in order to study the reactions and rearrangements of hop constituents.

For example, the most simple analogs of desoxyhumulone and lupulone are 3,5-dimethylphloracetophenone and 3,3,6-trimethylphloracetophenone. Both of these compounds are formed during methylation of phloracetophenone with methyl iodide under basic conditions. The amounts of these products can be determined rapidly by hplc (Figure 9). Oxidation of the dimethylphloracetophenone to 3,5-dimethyl-3-hydroxyphloracetophenone, an analog of humulone, is achieved by shaking with lead acetate in an atmosphere of oxygen. This reaction can also be followed by hplc, the starting material and product being rapidly and completely separated, as shown in Figure 9.

Alkylation of phloracylphenones with isopentenyl bromide also yields a mixture of products but an added complication exists in that cyclization of the isopentenyl sidechain to give a 2,2-dimethylchroman is quite facile even

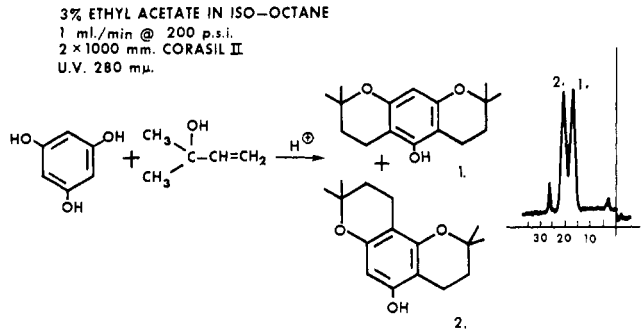


Figure 11. Separation of linear and angular phloroglucinol dichromans.

under mildly acidic conditions (Molyneux and Jurd, 1970). Thus, 3-(α,α-dimethylallyl)-phlorisovalerophenone cyclizes to give a mixture of two isomers, 5,7-dihydroxy-2,2-dimethyl-6-isovalerylchroman and 5,7-dihydroxy-2,2-dimethyl-8-isovalerylchroman, which are separable only with difficulty by fractional crystallization. Separation by hplc can be accomplished quite readily, however, and an estimate can be made of the quantity of each isomer formed (Figure 10).

A similar problem exists in the case of the linear and angular dichromans shown in Figure 11. The two isomers have similar melting points and can only be separated by repeated crystallization. Hplc on Corasil II using 4% ethyl acetate in isooctane gives reasonable separation and provides a rapid method for monitoring the purity of each isomer.

SUMMARY

The above examples demonstrate the application of hplc to one area of natural products chemistry. The technique can be used to study natural mixtures, to follow structural transformations of such compounds, and to monitor and separate mixtures obtained in synthetic studies. In the particular problem of concern to us, namely the hop resin bittering constituents, the technique has shown considerable utility but requires further development to attain its full potential. For example, it would be highly desirable to be able to separate the homologs of the α and β acid groups, but the major achievement would be the separation of the multitude of hop-derived flavor constituents present in beer. Undoubtedly, further refinements of the technique, such as gradient elution and use of high-pressure ion-exchange chromatography, will enhance the possibility of attaining these goals.

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Bitter Tasting Compounds of Beer. Chemistry and Taste Properties of Some Hop Resin Compounds

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The subject of beer flavor is presented and a description of the aroma and taste properties is given. Importance of studying the taste character of beer is stressed and a brief review is presented of the chemistry and taste properties of some nonvolatile resin constituents of hops. Some results are presented on the application of ion-exchange chromatographic and high-pressure liquid chromatographic analysis for the separation of hop resin compounds present in beer as well as in some samples representing different parts of the brewing process. The ion-exchange chromatographic procedure, while capable of resolving some beer bitter compounds, suffers from being a slow procedure with the possibilities of producing artifacts when working with the rather unstable group of hop compounds. The high-pressure liquid chromatographic procedure appears to have great potential in the analysis of hop bitter compounds. The method is rapid and produces a good resolution of hop compounds.

The flavor of beer, like that of many foods and beverages, is composed of many volatile and nonvolatile compounds present in a definite blend. Modern separation and identification techniques place the number of volatile and nonvolatile compounds in beer close to 400. It is reasonable to suppose, however, that only a small number of these compounds are "flavor active," that is to say, directly involved in producing the flavor sensation when the product is consumed.

The aroma of beer consists mainly of the sweet and pleasing note of esters, harsh tingling sensation of alcohols, characteristic aroma of aldehydes, ketones, and mercaptans, sour note of lower organic acids, and the indescribable yet pleasing note produced by hitherto unidentified compounds present in beer. Several reviews are available on the general composition of beer[11,28,32,42] but only a limited amount of work has been reported concerning the actual flavor influence of some of the beer constituents.[35-37] The complex nature of beer aroma still leaves it inadequately understood.

The taste aspect of beer has not been studied as extensively as the aroma. One of the reasons for this may be that the phenomenon of taste is considered to be much simpler than that of aroma. When applied to beer, the view of the taste phenomenon represents an oversimplification of the situation. Although generally, three basic tastes, namely sweet, sour, and bitter notes, are recognized in beer, the nature of beer constituents is such as to modify these tastes in a unique way. In addition, beer taste experience includes mouthfeel factors such as smoothness, harshness, and astringency which contribute to the overall characteristic sensation of beer taste. The object of this paper is to present a brief review of the chemistry and taste properties of some hop resin-derived compounds in beer. Results of ion-exchange and high-pressure liquid chromatographic tecnhiques for the separation of nonvolatile hop constituents in beer will also be reported.

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SOURCES OF TASTE COMPOUNDS IN BEER

In Table I are listed types of compounds believed responsible for the various taste notes in beer. Although a number of beer constituents such as polypeptides, proteins, and high molecular weight carbohydrates contribute to the bitter taste of beer, hop resins are considered to be by far the greatest contributors to this important property of beer. The bitter tasting compounds of hops are now known to be formed during the kettle boiling step in the manufacture of beer.

BITTER RESINS OF HOPS, THEIR CHEMISTRY AND DERIVATION INTO BEER

Hop resins, or the so-called bitter principles of hops, are present in the lupulin glands of the cones of female hop flowers. The hop plant (*Humulus lupulus*) is a climbing herbaceous plant belonging to the natural family of Maraceae and the natural order of urticales. The lupulin glands containing the bitter resins and essential oil are secreted at the base of the female flowers (Figure 1). The hop petals contain polyphenolic compounds. The hop polyphenols, which constitute about 4% of the hop cone, also enter into some reactions during the brewing process and are believed responsible for imparting an astringent taste to beer, but this aspect of hops will not be discussed here.

A typical composition of hop cone is shown in Table II.

Hop resins, which account for about 15% of the hop cone (dry weight), consist of several distinct compounds. Early work on the fractionation of hop resins,[14,15] which was based on the solubility of resins in various organic solvents, classified them into soft resins (α and β acids and uncharacterized soft resins) and hard resins (xanthohumol, oxidized resins). At that time α and β acids were believed to represent single compounds but subsequent